Placental syncytins: Genetic disjunction between the fusogenic and immunosuppressive activity of retroviral envelope proteins

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We have previously demonstrated that the envelope proteins of a murine and primate retrovirus are immunosuppressive *in vivo*. This property was manifested by the ability of the proteins, when expressed by allogeneic tumor cells normally rejected by engrafted mice, to have the *env*-expressing cells escape (at least transiently) immune rejection. Here, we analyzed the immunosuppressive activity of the human and murine syncytins. These are envelope genes from endogenous retroviruses independently coopted by ancestral hosts, conserved in evolution, specifically expressed in the placenta, and with a cell–cell fusogenic activity likely contributing to placenta morphogenesis. We show that in both humans and mice, one of the two syncytins (human syncytin-2 and mouse syncytin-B) is immunosuppressive and, rather unexpectedly, the other (human syncytin-1 and mouse syncytin-A) is not (albeit able to induce cell–cell fusion). Delineation of the immunosuppressive domain by deletion analysis, combined with a comparison between immunosuppressive and nonimmunosuppressive sequences, allowed us to derive a mutation rule targeted to specific amino acids, resulting in selective switch from immunosuppressive to nonimmunosuppressive envelope proteins and vice versa. These results unravel a critical function of retroviral envelopes, not necessarily ''individually'' selected for in the retrovirus endogenization process, albeit ''tandemly'' conserved in evolution for the syncytin pairs in primates and Muridae. Selective inactivation of immunosuppression, under conditions not affecting fusogenicity, should be important for understanding the role of this function in placental physiology and maternofetal tolerance.

endogenous retrovirus | fusogenicity | immunosuppression | HERV

The placenta is an autonomous and transient organ essentially intended for feeding and oxygenating the embryo and the fetus during intrauterine life. In several mammalian species, including *Homo sapiens*, the fusion of trophoblastic cells into a multinucleated layer called syncytiotrophoblast constitutes a key process of placental morphogenesis. The syncytiotrophoblast, being the main maternofetal barrier in direct contact with maternal blood, performs the essential trophic exchange functions between mother and fetus, along with the secretion of hormones and growth factors, maintenance of homeostasis, and the necessary inhibition of the mother's immune response against the allogeneic determinants of the fetus (1–3).

Little is known about the molecular mechanisms involved in trophoblastic differentiation. However, a major advance has been made by the identification of envelope (Env) proteins encoded by endogenous retroviruses (ERVs) and likely involved in the formation of the syncytiotrophoblast (4–7). The human and murine genomes indeed harbor thousands of ERV elements that display a structure close to that of the integrated proviral form of exogenous retroviruses and that most probably are the remnants of past infections of the germ line by ancestral retroviruses (8–11). Although the vast majority of these elements are defective, a few of them still contain intact ORFs, notably in *env* genes. During the retroviral life cycle, Env glycoproteins, which are anchored in the lipid bilayer of viral surface envelopes, are involved first in cell surface receptor recognition and next in viral entry by driving the fusion of the viral envelope with the cell membrane. Cell surface expression of Env proteins in isolation can trigger cell–cell fusion provided that their cognate receptors are exposed at the surface of neighboring cells. A systematic search through the human genome sequence has identified 18 coding *env* genes, whose products may potentially have a function (4, 12–14). Among them, the syncytin-1 and -2 proteins are specifically expressed within the placenta at the cytotrophoblast–syncytiotrophoblast interface and have been reported to induce cell–cell fusion *ex vivo* by interacting with distinct receptors (4–6, 15, 16). Syncytin-1 was shown to be directly involved in the differentiation and fusion of human cytotrophoblasts in primary cultures (5, 6, 16). In addition, the functionality of these two genes has been conserved in evolution since the time of their insertion into the primate genome (some 25–40 Myr ago), and they currently display remarkably little polymorphism in the human population (4, 17, 18), two strong signs of purifying selection. The host has most probably coopted these genes of retroviral origin for its own benefit, more specifically for syncytiotrophoblast morphogenesis.

A second hypothetical function of syncytins is related to their putative immunosuppressive (IS) activity. This hypothesis relies essentially on the presence within retroviral Envs of a conserved domain, located within their transmembrane (TM) subunit, which was initially shown to have IS effects in *ex vivo* assays (19). These results were further substantiated by a series of experiments carried out *in vivo* in which we have shown that the expression of the Env protein of a murine leukemia virus (MLV) (20), of the simian Mason–Pfizer Monkey Virus (MPMV) (21), and of a human endogenous retrovirus (HERV) of the HERV-H family (22) can antagonize the immune-system-dependent elimination of tumor cells injected into immunocompetent mice after transduction of these cells by an Env-expression vector. Consistently, and based on the fact that the placenta, expressing both maternal and paternal antigens, can be considered as a semiallogeneic graft that needs to be tolerated by the mother, an IS activity of the syncytin genes (that would be involved in maternofetal tolerance) has been suggested but never demon-

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strated. Noteworthily, such a role does not require Env proteins to be fusogenic, and this is the case for a third, almost complete retroviral *env* gene [namely, ERV-3 *env* (13)] that is truncated before the transmembrane domain and thus unable to induce cell–cell fusion, but that still contains the putative IS domain (ISD).

Here, we have analyzed the IS activities of a series of retroviral Envs, including syncytin-1, syncytin-2, and ERV-3 Env, and found rather unexpectedly that, whereas syncytin-2 and ERV-3 Env are immunosuppressive, syncytin-1 clearly is not. Furthermore, based on sequence comparison between the homologous domains of syncytin-1 and of MPMV Env, we identified a key amino acid responsible for the lack of IS activity of syncytin-1. Proper reciprocal amino acid substitutions allowed us to confer an IS capacity to syncytin-1 and, conversely, to abolish that of MPMV Env. One remarkable feature of the identified substitutions is that the primary function of these Env proteins (i.e., their virus–cell and/or cell–cell fusogenic activity) was not impaired. These results demonstrate that the IS function of retroviral Envs can be uncoupled from their basic "mechanical" functions associated with membrane fusion and substantiate the hypothesis that syncytin-2 and ERV-3 Env, by means of their IS activity, could play a role in fetomaternal tolerance. Similar results obtained with the mouse genes *syncytin-A* and *-B*, which are homologous but not orthologous to the human *syncytin-1* and *-2* genes (23), further strengthen this hypothesis.

Results

Immunosuppressive Properties of Syncytin-1 and -2. The IS properties of the two HERV-encoded human syncytins (namely, syncytin-1 and -2) were analyzed by using an *in vivo* tumor-rejection assay that we had previously used to demonstrate the IS activity of the Env protein of the Moloney murine leukemia virus (MoMLV) and the MPMV retrovirus, as well as of the MoMLV TM subunit alone (see Env structure in Fig. 1*A*) (20, 21). The rationale of the assay (see Fig. 1*B*) can be summarized as follows: whereas injection of MCA205 tumor cells (H-2^b) into allogeneic BALB/c mice (H-2^d) leads to the formation of no tumor or transient tumors that are rapidly rejected, injection of the same cells but stably expressing an immunosuppressive retroviral Env leads to the growth of larger tumors that persist for a longer time, despite the expression of the new exogenous antigen. This difference is not associated with a difference in intrinsic cell growth rate because it is not observed in syngeneic C57BL/6 mice and is immune system dependent. The extent of "immunosuppression" can be quantified by an index based on tumor size: $(A_{\mathrm{env}} - A_{\mathrm{none}})/A_{\mathrm{none}}$, where A_{env} and A_{none} are the mean areas at the peak of growth of tumors from BALB/c mice injected with *env*-expressing or control cells, respectively. A positive index indicates that *env* expression facilitates tumor growth, as a consequence of its IS activity; a null or negative index points to no effect or even an inhibitory effect, respectively. The latter may be explained by a stimulation of the immune response of the host against the new foreign antigen, represented by a non-IS Env, expressed at the surface of the tumor cells.

MCA205 cells were therefore transduced with expression vectors for each syncytin and assayed for tumor cell growth upon both syngeneic (control) and allogeneic engraftment. As expected and observed for the control in Fig. 1*D*, the syncytin-1 and -2 transduced MCA205 cells had identical growth rates as compared with the MCA205 control cells upon injection into syngeneic mice. In the tumor-rejection assay using the same cells in allogeneic mice (Fig. 1*C*), the MCA205 cells expressing syncytin-2 formed easily detectable and long-lasting tumors in 100% of the engrafted animals—at variance with those detected with the control MCA205 cells—thus indicating an IS activity *in vivo* and resulting in a positive immunosuppression index (Fig.

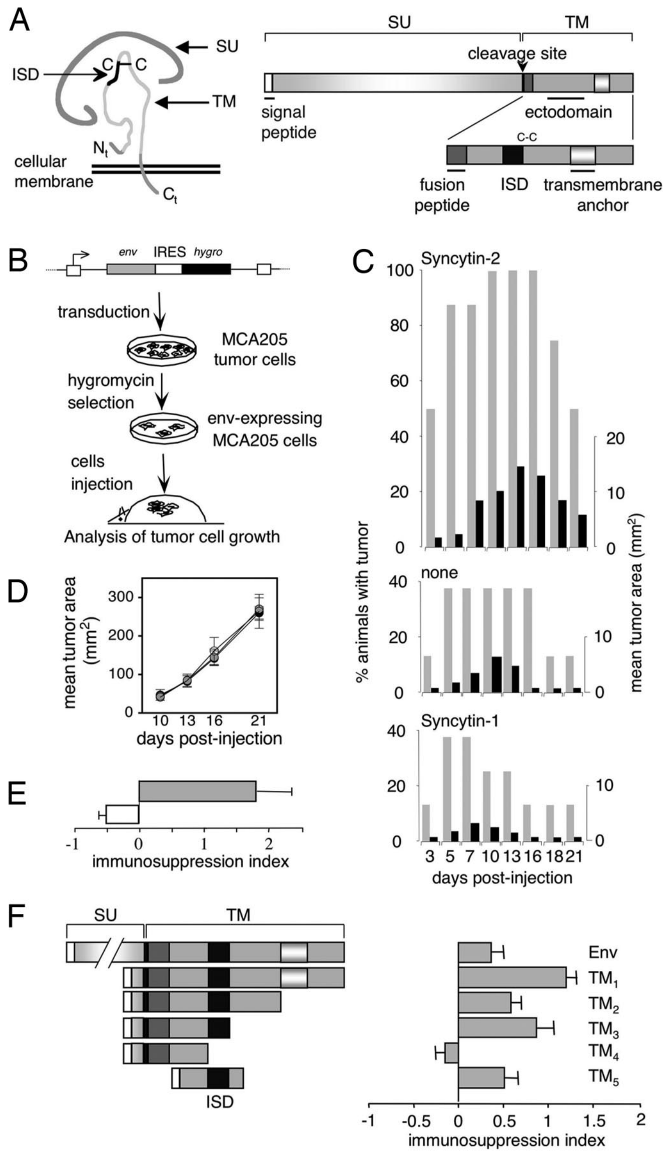

Fig. 1. Analysis of the immunosuppressive activity of syncytins and of retroviral envelope subdomains. (*A*) Schematic representation of a retroviral envelope protein with its surface (SU) and transmembrane (TM) subunits and ISD. (*B*) Rationale of the *in vivo* immunosuppression assay. MCA205 tumor cells transduced with the *env*-expressing pDFG vector were selected in hygromycin-containing medium. The Env-expressing cells were then injected s.c. into mice, and tumor growth was monitored. (*C*) Immunosuppressive activity of syncytin-1 and -2. MCA205 cells transduced with the pDFG vector encoding the syncytin-1 and -2 *env* or an empty vector (none) were injected into allogeneic BALB/c mice (10^6 cells per mice, 8 mice per group), and tumor size was measured. Shown are the percentages of animals with tumor (gray bars) and mean tumor areas when >1 mm^2 (black bars). (*D*) Control growth of the MCA205 cells in *C* (black circles, syncytin-1; gray circles, syncytin-2; open circles, none) injected into syngeneic C57BL/6 mice; mean tumor areas ± SD are indicated (10^6 cells per mice, 3–5 mice per group). (*E*) Immunosuppressive indexes (see text) of syncytin-1 (open bar) and syncytin-2 (gray bar) (same experimental conditions as in *C*; means ± SD, $n = 3$). (*F*) Functional delineation of the ISD of MoMLV Env. The IS activity of full-length and truncated/deleted envelopes (structures at *Left*) was tested by using the MCA205 tumor-rejection assay as in *B*–*D* (immunosuppression indexes at *Right*; means ± SD, $n = 3$).

1*E*). This result is shared by all of the retroviral Envs that we had previously tested with this assay (20–22). Surprisingly, the syncytin-1 transduced MCA205 cells formed tumors of a reduced size, which were detected only in a limited fraction of the engrafted animals and were rapidly eliminated, thus giving a negative index indicative of a lack of IS activity (Fig. 1*E*). Noteworthily, syncytin-1 is the first Env protein identified so far to be deprived of an IS activity, despite the conservation of its fusogenic property. Finally, we attempted to determine the IS activity of the HERV-encoded ERV-3 protein, also expressed in the placenta (but not fusogenic). Unfortunately and in accordance with Lin *et al.* (24), expression of ERV-3 Env was found to inhibit the *in vitro* proliferation of the transduced cells (data not shown), thus barring analysis of its IS function (but see below for a discussion of the IS activity of its ectodomain).

Identification of the Determinants of the Immunosuppressive Activity of Retroviral Env Proteins. The syncytin-1 result led us to hypothesize that immunosuppression might be dissociated from the usual "mechanical" functions of retroviral Envs—i.e., be specifically abolished by mutations that would concurrently leave fusogenicity intact. We set out to identify a potential "signature" of the IS activity within the sequence of Env proteins, by comparison with that of syncytin-1. To be practical, this comparison had to give the least number of differences—hence be performed within the smallest possible domain still able to induce immunosuppression *in vivo*, and with the IS Env the most similar to syncytin-1 across this domain.

To first delineate a minimal ISD active *in vivo*, we analyzed the effect of a series of truncations/deletions within the *env* gene of the model MoMLV retrovirus. The series of truncations in Fig. 1*F* identified a 20-residue-long ISD, overlapping a central loop connecting two α-helices in the TM subunit and comprising the 17 amino acids of the previously characterized CKS-17 peptide (19). The ISD is embedded into the so-called ectodomain, which corresponds to a soluble part of the extracellular domain of the TM subunit and has boundaries close to those of the previously crystallized and highly structured domains of several retroviral Envs (25–27) (see Fig. 2*B*). As illustrated in Fig. 1*F* with the TM_5 construct, this structured domain retains the IS activity of the entire Env protein.

We next searched for an IS retroviral Env containing an ISD with a strong sequence similarity to that of syncytin-1. Interestingly, as illustrated in Fig. 2*A*, the ISD of syncytin-1 shares 80% identity with that of MPMV Env that we had previously shown to be immunosuppressive in the MCA205 assay (21). On the basis of this amino acid comparison, we first looked for mutations that could selectively confer an IS activity to syncytin-1, without affecting its fusogenic function. We individually replaced each of the four discriminating residues in the syncytin-1 ISD—alanine (A), arginine (R), threonine (T), and phenylalanine (F)—at position 6, 14, 17, and 20, respectively, with that found at the identical position in the ISD of MPMV Env—glycine (G), glutamine (Q), isoleucine (I), and alanine (A), respectively (Fig. 2*A*). Two of these mutations (A6G and T17I) had no effect on syncytin-1 fusogenicity, but neither did they affect immunosuppression (Fig. 2 *C* and *E*). The two others (i.e., R14Q and F20A) significantly affected fusogenicity, suggesting that they were disrupting the expression, folding, or trafficking of syncytin-1. This prevented us from testing whether these substitutions might confer an IS activity to syncytin-1. In contrast, introduction of the reverse mutation, Q14R, into MPMV Env did not affect the infectivity of pseudotyped MLV virions (Fig. 2*D*). However, remarkably, the Q14R mutation abolished the MPMV Env IS activity (Fig. 2*E*). Thus, the IS activity of MPMV Env critically depends on the identity of the amino acid found at position 14 of the ISD. To cope with the loss of fusogenicity caused by the reciprocal R14Q mutation observed above with syncytin-1, we first derived a structural model for the syncytin-1 ectodomain and ISD based on its homology with that of MoMLV Env. It shows that the residues found at positions 14 and 20 are spatially close and could be in contact, the electro-

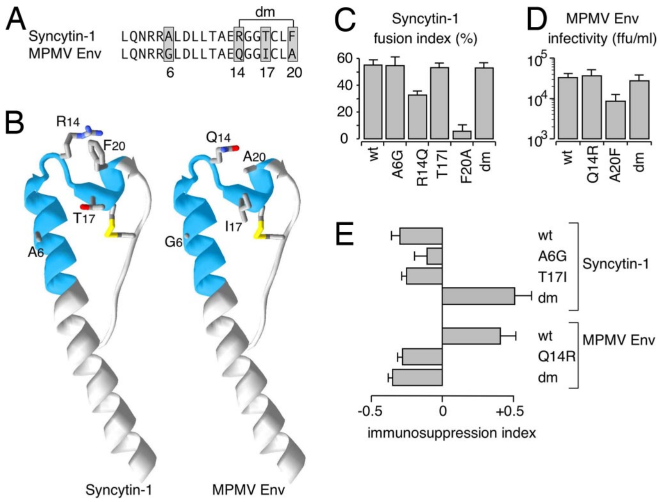

Fig. 2. Identification of the mutations selectively affecting the IS activity of retroviral Env proteins. (*A*) Aligned sequences of the ISD of syncytin-1 and MPMV Env, with the four divergent residues grayed. The bracket links the two key residues reciprocally substituted in the double-mutant (dm) constructs. (*B*) Model structures of syncytin-1 (*Left*) and MPMV Env (*Right*) ectodomains calculated from the molecular structure of the syncytin-2 ectodomain previously determined by x-ray crystallography (27). Side chains are represented only for the four divergent residues. (*C*) Cell–cell fusion activity of syncytin-1 (wild-type, wt) and its mutant derivatives in HeLa cells. Fusion indexes were determined as indicated in *Materials and Methods* (means ± SD, $n = 3$). (*D*) Infectivity of MPMV Env and its mutant derivatives as expressed on the surface of MLV viral pseudotypes, using HeLa cells as a target. Titers (LacZ-positive focus forming units) were measured as in ref. 32 (means ± SD, $n = 3$). (*E*) *In vivo* IS activity of syncytin-1 and MPMV Env and their mutant derivatives. Same experimental conditions as in Fig. 1*E*.

negative phenyl ring of phenylalanine, F20, most probably interacting with the positive charge of arginine, R14 (Fig. 2*B*). This suggested that the bulky side chain of F20 might be incompatible with Q14 in place of R14 and that reciprocal exchange of F20 to A20 should accompany the R14Q mutation to recover both a proper conformation and fusogenicity. Indeed, as observed in Fig. 2*C*, the R14Q + F20A double mutation (dm) both preserved syncytin-1 fusogenicity and conferred IS activity (Fig. 2*E*). In conclusion, the IS activity of the retroviral Env protein seems to depend primarily on the amino acid at position 14 of the ISD, with arginine being the signature of an Env devoid of IS activity, whereas conservation of the fully active Env further requires the concomitant mutation of the amino acid at position 20.

Selective Modulation of the Immunosuppressive Properties of Retroviral Env Proteins. To evaluate further the importance of the key residue at position 14 of the ISD for the IS activity of the Env proteins expressed in the placenta, including the ERV-3 Env, we constructed expression vectors for 63- or 64-residue-long fragments of the TM subunits that correspond to the ectodomain, including the ISD (see Figs. 1*A* and 2*B*). Such ectodomains are large enough to adopt a proper conformation (see, for instance, the crystallographic structure of the syncytin-2 ectodomain and of other retroviral Envs in refs. 25–27). We first confirmed, as demonstrated in Fig. 1*E* for MoMLV, that expression of the syncytin-2 ectodomain is sufficient to induce immunosuppression in the tumor-rejection assay (Fig. 3 *A* and *B*). Under the same conditions, the syncytin-1 ectodomain remained nonimmunosuppressive (Fig. 3 *A* and *B*). Interestingly, expression of the ectodomain of ERV-3 Env, in contrast to that of the full-length protein, did not inhibit cell proliferation either *in vitro* or *in vivo* in the syngeneic control engraftment (data not shown),

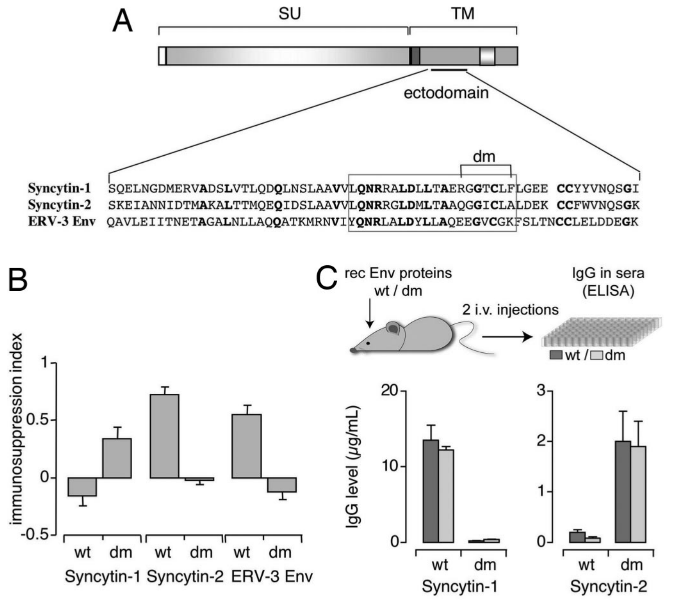

Fig. 3. Immunosuppressive properties of human placental Env proteins. (*A*) Alignment of the TM subunit ectodomain sequences from syncytin-1, syncytin-2, and ERV-3 Env. The ISD is framed, and the bracket indicates the positions mutated to generate the double-mutant (dm) derivatives. (*B*) *In vivo* IS activity of the ectodomain of the wild-type (wt) syncytin-1, syncytin-2, and ERV-3 Env, and their mutant (dm) derivatives. Immunosuppression indexes for the ectodomains were measured as in Fig. 1 (means ± SD, $n = 3$). (*C*) Immunosuppressive activity of syncytins as revealed by the differential antibody response to the wild-type and mutant recombinant ectodomains. Swiss mice (five animals per experiment) were immunized twice with either wild-type or double-mutant recombinant ectodomains (100 μg per i.v. injection). Serum levels of IgG antibodies directed against the ectodomain of each protein were assayed on ELISA plates coated with either the wild-type (dark gray) or mutated (light gray) corresponding ectodomains (mean values ± SD, $n = 3$).

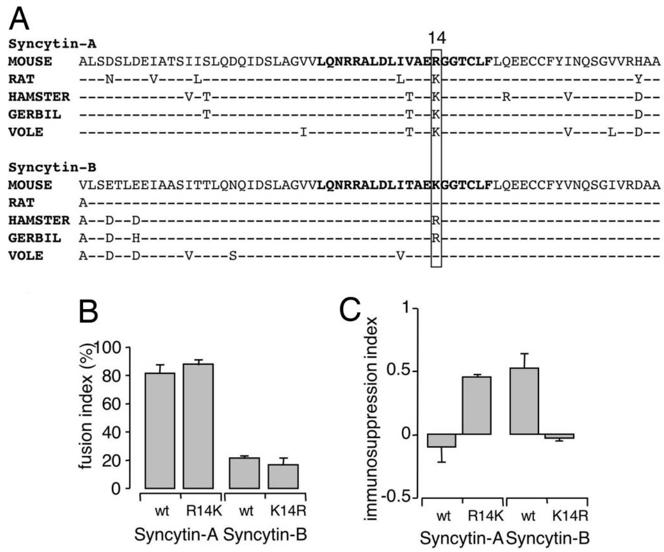

Fig. 4. Sequence alignment of rodent syncytin-A and -B, and IS properties of the murine syncytins. (*A*) Alignment of the TM subunit ectodomain sequences from murine syncytin-A and -B with those of the orthologous proteins from several rodents (GenBank entries AY849973 through AY849981). Only the residues differing from the murine sequences are indicated, with the ISD residues in boldface and the key 14th residue boxed. (*B*) Cell–cell fusion activity of the murine syncytin-A and -B and their mutant derivatives, in 293T and A23 cells, respectively (mean values ± SD). (*C*) *In vivo* IS activity of the murine syncytin-A and -B ectodomains and their mutant derivatives. Same experimental conditions as in Fig. 3*B* (mean values ± SD).

thus enabling demonstration of its IS activity (Fig. 3 *A* and *B*). We then mutated to arginine (as found in syncytin-1) the glutamic acid or glutamine at position 14 of the ISD of the ERV-3 and syncytin-2 ectodomains, respectively. Simultaneously, the residues at position 20 (lysine in ERV-3 or alanine in syncytin-2 ISD) were replaced by phenylalanine to restore the putative interaction with arginine 14 that appeared to be necessary to preserve the structural integrity and the fusogenic function of syncytin-1 (see above). The reciprocal mutations that have been described above for the full-length syncytin-1 (i.e., the double mutant R14Q + F20A) were also introduced into its 64-aa ectodomain. As illustrated in Fig. 3*B*, these definite double mutations resulted in (*i*) inhibition of the IS activity of the syncytin-2 and ERV-3 ectodomains and, conversely, (*ii*) induction of IS activity in the otherwise immunosuppression-negative syncytin-1 ectodomain. As a control for the structural integrity of the syncytin-2 double mutant, the same mutations were introduced into the full-length syncytin-2 and found not to impair its fusogenic function (fusion index of 85 ± 6% for wild-type and 84 ± 2% for mutant syncytin-2).

These results were further extended to two other possibly physiologically relevant HERV Envs—namely, the placenta-specific (but not fusogenic) HERV-V and the fusogenic (but not placenta-specific) HERV-P(b) (12, 14). Expression vectors for their ectodomains and double-mutant derivatives were constructed [see supporting information (SI) Fig. 5] to assay immunosuppression *in vivo*. Interestingly, as shown in SI Fig. 5, both Envs proved to be immunosuppressive, and reversion of this function was observed for the double mutants.

Immunosuppressive Properties of Syncytins as Revealed by Antibody Response to Recombinant Ectodomains. The MCA205 tumor-rejection assay essentially measures the ability of the IS Env proteins to inhibit a cellular immune response. To investigate whether the IS activity of placental Env proteins could also inhibit a humoral Env-targeted immune response, we designed an assay based on the analysis of antibody production by mice injected with ISD-containing recombinant Env ectodomains. The recombinant ectodomains of syncytin-1 and -2, in their wild-type and double-mutant forms, were produced in *Escherichia coli* as soluble and folded His-tagged trimers and purified. Each protein was injected i.v. twice at a 1-week interval into Swiss mice, sera were collected 4 days after the last injection, and IgG titers were assayed by ELISA. As illustrated in Fig. 3*C*, the non-IS form of each ectodomain (wild-type syncytin-1 and double-mutant syncytin-2) induced IgG titers 10- to 30-fold higher than the corresponding IS form (mutant syncytin-1 and wild-type syncytin-2). Importantly, in both cases, the antibodies induced by the injection of a given ectodomain form recognized equally well both the wild-type and double-mutant forms [identical IgG titers for wild-type- and double-mutant-coated microplates (Fig. 3*C*)], indicating that the higher humoral response obtained with the non-IS ectodomains did not result from the fortuitous generation of a novel dominant epitope by the introduced mutations. Clearly, the IS activity of the Env proteins as revealed *in vivo* in the tumor-rejection assay can also be detected by its inhibiting effect on the Env-targeted antibody response.

Murine Placental Env Proteins Homologous to Human Syncytins Share the Same Immunosuppressive Properties. Syncytin-A and -B are two recently identified murine ERV *env* genes homologous, but not orthologous, to the human syncytin genes (i.e., they were independently coopted by rodent ancestors), which are specifically expressed in the placenta and encode full-length fusogenic Envs (23). As illustrated in Fig. 4*A*, the syncytin-A ISD displays the signature of a non-IS Env protein, with R at position 14 and F at position 20, the same amino acid pair as in the immunosuppression-negative human syncytin-1. Conversely, syncytin-B has lysine (K) at position 14 and F at position 20. This combination is also found in the HERV-H Env protein previously reported to be immunosuppressive (22), indicating that this pair should be compatible with an immunosuppressive and structurally relevant Env protein. Hence, we analyzed the IS activity of the murine syncytins, both the wild-type forms and those obtained after introduction of the K14R mutation into syncytin-A and of the R14K mutation into syncytin-B. We first checked that these modifications did not alter their fusogenic properties, as illustrated in Fig. 4*B* for the wild-type and mutant Envs. We then performed the MCA205 tumor-rejection assay, with the corresponding wild-type and mutant ectodomains, as above in Fig. 3*B* for the human syncytins. As illustrated in Fig. 4*C*, the wild-type syncytin-B ectodomain is immunosuppressive. This activity is specifically abolished in the K14R mutant, thus indicating that the K14 signature within the ISD is critical for the IS activity. In the case of the syncytin-A ectodomain, we show that it is not immunosuppressive but that, as expected, it could be rendered so by the reverse R14K mutation (Fig. 4*C*). The murine genome thus harbors (as does the human genome) two syncytin genes, among which one encodes an immunosuppressive protein. In addition, *in silico* analysis suggests that, among the four other rodents whose genes were examined, at least one of the two orthologous syncytin genes encodes an Env protein with an IS signature, i.e., a lysine at ISD position 14 (Fig. 4*A*). In the case of the rat and the vole, both syncytins harbor the IS K14 signature.

Discussion

Placenta has long been viewed as a semiallogeneic graft able to invade part of the mother uterine tissue and responsible for tolerance of the fetopaternal antigens by the mother immune system. Along these lines, it has been tempting to hypothesize that retroviruses, which are in most cases both oncogenic and immunosuppressive, have been founder elements in the evolutionary passage from egg-laying to placental mammals, some 100 Myr ago (2, 28–30). Actually, it is well known that retroviruses have parasitized living species well before the emergence of mammals, and the traces of these ancient invasions can be found in the genome of most present-day living species (8–11). Examples of retroviral infections resulting in their endogenization and capture for the host benefit are numerous and include the acquisition of envelope genes of retroviral origin with specific expression in the placenta (4, 7, 17, 18, 23): syncytin-1 and -2 in primates and syncytin-A and -B in Muridae, all of which have then been conserved in a functional state as bona fide genes with a placenta-specific expression, with in all four cases evidence for purifying selection in the course of evolution. A first important issue of the present investigation is the demonstration that in both primates and Muridae, at least one of the syncytins is immunosuppressive: syncytin-2 in humans, which is the most ancient one, being present in all primates except prosimians (i.e., >40 Myr), and syncytin-B in the mouse. This demonstration was achieved by using a physiologically relevant *in vivo* assay, whereby expression of the immunosuppressive syncytin proteins allowed the transient engraftment of otherwise rejected allogeneic tumor cells. The same assay had previously been used to demonstrate IS activity of the EBV vIL-10 protein (31) and of the Envs of several retroviruses (20–22). IS activity of syncytin-2 was further assessed by another *in vivo* assay carried out by using recombinant Env subdomains (namely the ectodomains), which demonstrated enhancement of the antibody response against the Env proteins after specific inactivation of the ISD function (see below). Noteworthily, analysis of the sequences of the ISD of syncytin-2 in all primates where the gene is present and has been sequenced (32) discloses 100% amino acid conservation, thus strongly suggesting conservation of the IS function. One hundred percent amino acid conservation also holds for the syncytin-1 ISD (17) but with an immunosuppression-negative signature. In Muridae, the situation is slightly different, albeit very illustrative, with a marked sequence polymorphism for both syncytin-A and -B at amino acid position 14 of the ISD, which we have shown to be critical for IS activity: the K to R substitution in the mouse syncytin-B is deleterious for immunosuppression, and the reverse R to K substitution within the otherwise immunosuppression-negative mouse syncytin-A confers IS activity. Remarkably, analysis of this key position within the Muridae syncytin-A and -B genes (Fig. 4*A*) discloses that for each animal branch, at least one (for the mouse, hamster, and gerbil) and in some cases both (rat and vole) syncytins carry the immunosuppression-positive K14 signature, strongly suggesting functional "complementation" in the course of evolution between the two syncytins.

In the case of the other HERV Envs that we have tested in addition to syncytin-1 and -2, namely the ERV3, HERV-P(b), and HERV-V Envs, all were found to be immunosuppressive, although they each lack at least one of the canonical properties of bona fide syncytins: ERV3 and HERV-V Envs are specifically expressed in the placenta but are neither fusogenic nor strictly conserved in evolution, and the HERV-P(b) Env is not expressed at a significant level in the placenta (12–14). Although one cannot exclude that the IS activity of at least two of these proteins participates (or has participated), concomitantly with syncytin-2, in placenta physiology, a more likely interpretation could be that they simply are "degenerate syncytins": Along this line, the immunosuppressive and fusion-negative ERV3 *env* gene discloses a strong polymorphism in the human population, with even 1% of Caucasians being homozygous for a stop mutation severely truncating the protein and eliminating de facto the ISD (33). In addition, the ERV3 *env* gene is lost in gorilla (18). Accordingly, retroviral envelope-mediated immunosuppression in the placenta might not necessarily be redundant, and this function has possibly been fulfilled in the course of evolution by distinct retroviral Envs, with possible "transient" redundancy periods.

A second major outcome of the present investigation is the identification of immunosuppression-negative ISD among functional, fusogenic Env proteins, namely human syncytin-1 and mouse syncytin-A, which demonstrates that the IS function can be uncoupled from the primary "mechanical" fusogenic function of retroviral Envs. In turn, this allowed us to (*i*) unravel the molecular determinants of the IS activity, with the precise identification of the critical amino acids responsible for this function, and (*ii*) switch on or off the IS activity of a series of retroviral Envs, under conditions that preserve their "mechanical" properties. The ISD overlaps a constrained segment of the Env protein that should play a pivotal role through its conformational changes during the fusion process. Its structure is highly conserved among both infectious and endogenous retroviruses with an almost perfect superposition of the crystallographic structures of the MoMLV, HTLV-1, and syncytin-2 ectodomains, as illustrated in ref. 27. As a consequence of its critical position, mutations inactivating the IS function while preserving fusogenicity had to take into account the interactions between spatially close, albeit nonadjacent, amino acids: We demonstrate that the specific inactivation of the IS function of syncytin-2 and, symmetrically, the induction of IS activity to the otherwise immunosuppression-negative syncytin-1 require the combined substitution of the amino acid at position 20 in addition to that at position 14. The latter is critical for the IS activity, as further demonstrated by the on- and off-switching that can be achieved in the case of the mouse syncytins by its sole substitution.

In conclusion, the present work, with the demonstration of the IS activity of Env proteins and its possible uncoupling from their primary fusogenic function after definite mutations opens new perspectives. It provides a model that should allow one to dissociate the role of fusogenicity in placental morphogenesis from that of immunosuppression in placental physiology and maternofetal tolerance, for instance by generating knock-in mice for the mutant *syncytin-B* gene with a disrupted IS function. Furthermore, specific disruption of the IS function of Env proteins from infectious retroviruses, through targeted mutagenesis of their ISD, should allow an unambiguous characterization of the role of immunosuppression *per se* in viremia, for successful retroviral infection *in vivo*.

Materials and Methods

Mice and Cell Lines. C57BL/6 and BALB/c mice, 10 weeks old, were obtained from Janvier. 293T (ATCC CRL11268), HeLa (ATCC CCL2), G355–5 (ATCC CRL2033), A23 [a gift from J. Weissenbach, Evry, France (34)], and MCA205 (35) cells were cultured in DMEM supplemented with 10% FCS (VWR), streptomycin (100 μg/ml), and penicillin (100 units/ml).

Plasmids. Plasmids pDFG-MoTM$_1$ (20), pDFG-none (20), phCMV-syncytin-1 (5), phCMV-syncytin-2 (4), phCMV-envERV-3, phCMV-envV (12), phCMVenvP(b), phCMV-syncytin-A (23), phCMV-syncytin-B (23), pCR2.1ph74 (5), pTMO (36), pET28(+)b (Novagen), and pSecTag2A (Invitrogen) have been described. The phCMV- and pDFG-based expression vectors for the full-length and truncated Envs, and the pET28(+)b-based plasmids for production of recombinant Env domains are described in *SI Materials and Methods*. Primer sequences are listed in SI Table 1.

Cell–Cell Fusion and Infectivity Assays. Syncytium formation and viral pseudotyping assays were performed as described in ref. 4, using the wild-type and mutant phCMV-Env expression vectors. Target cells were HeLa cells for syncytin-1 and MPMV Env, G355–5 cells for syncytin-2, 293T cells for syncytin-A, and MDCK cells for syncytin-B. The fusion index, which represents the percentage of fusion events in a cell population, is defined as $[(N - S)/T]/100$, where N is the number of nuclei in the syncytia, S is the number of syncytia, and T is the number of nuclei counted (32).

***In Vivo* Tumor-Rejection Assays.** 293T cells (7.5×10^5) were cotransfected with the *env*-expressing pDFG retroviral vector to be tested (1.75 μg) and expression vectors for the MLV proteins (0.55 μg for the amphotropic MLV *env* vector and 1.75 μg for the MLV *gag* and *pol* vector; see ref. 4). Thirty-six hours after transfection, supernatants were harvested for infection of MCA205 cells (2.5 ml per 5×10^5 cells with 8 μg/ml polybrene). Cells were maintained in selective medium (400 units/ml hygromycin) for 3 weeks and then washed with PBS, scraped without trypsinization, and inoculated s.c. in the mouse flank as described in ref. 20. Tumor area (mm^2) was determined by measuring perpendicular tumor diameters. Mice were maintained in the animal facility of the Gustave Roussy Institute in accordance with institutional regulations.

Immunogenicity Assays. Recombinant proteins were produced in BL21 (DE3) *E. coli* cells (Stratagene) by using the pET28(+)b-based expression vectors. The recombinant wild-type and mutant ectodomains (behaving as soluble materials) were purified on HiTrap Chelating HP columns (Amersham) and further filtered through Superdex 75 HR10/30 columns (Amersham) to isolate the major trimeric form. Their LPS content was measured with the LAL QCL-1000 kit (Cambrex) and made equal by addition of *E. coli* LPS (strain 0111:B4; Sigma). One hundred micrograms of each recombinant ectodomain were then injected twice at 1-week intervals into Swiss mice, and sera were collected 4 days after the last injection. IgG levels were assayed by indirect ELISA, using MaxiSorp microplates (Nunc) coated with each recombinant ectodomain (2 μg/ml), anti-mouse IgG antibodies conjugated to horseradish peroxidase (Amersham), and *o*-phenylenediamine as a chromogenic reagent (Sigma). Mouse anti-His$_5$ antibody (QIAGEN) was used to standardize the data.

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